

Allyl isothiocyanate triggers G2/M phase arrest and apoptosis in human brain malignant glioma GBM 8401 cells through a mitochondria-dependent pathway

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Received February 12, 2010; Accepted April 16, 2010

DOI: 10.3892/or_00000878

Abstract. Isothiocyanates (ITCs) are present as glucosinolates in various cruciferous vegetables. Allyl isothiocyanate (AITC) is one of the common naturally occurring isothiocyanates. Recent studies have shown that AITC significantly inhibited survival of leukemia HL-60, bladder cancer UM-UC-3 and colon cancer HT-29 cells *in vitro*. In this study, we demonstrate that AITC significantly decreased proliferation and viability of human brain malignant glioma GBM 8401 cells in a dose-dependent manner with IC₅₀ 9.25±0.69 μM for 24 h-treatment. The analysis of cell cycle distribution also showed that AITC induced significantly G2/M arrest and sub-G1 phase (apoptotic population) in GBM 8401 cells. AITC markedly reduced the CDK1/cyclin B activity and protein levels by CDK1 activity assay and Western blot analysis. AITC-induced apoptotic cell death and this evidence was confirmed by morphological assessment and DAPI staining. Pretreatment with specific inhibitors of caspase-3 (Z-DEVE-FMK) and -9 (Z-LEHD-FMK) significantly reduced caspase-3 and -9 activity in GBM 8401 cells. Western blot analysis and colorimetric assays also displayed that AITC caused a time-dependent increase in cytosolic cytochrome *c*, pro-caspase-9, Apaf-1, AIF, Endo G and the stimulated caspase-9 and -3 activity. Our results suggest that AITC is a potent anti-human brain malignant glioma drug and it shows a remarkable action on cell cycle arrest before commitment for apoptosis is reached.

Introduction

Glioblastoma multiforme (GBM) is a common and lethal primary malignant brain tumor (1). In the primary GBM, the 2-year survival rate is <5% (2). During the last two decades, many treatments for GBM, including surgical therapy, radiotherapy and chemotherapy were suggested. However, the GBM survival rate has not changed (3). With the mechanisms of GBM revealed gradually (4), treatments have been focused on reducing tumor growth or inducing apoptosis mechanisms to improve survival. So far, the therapy for brain tumors in clinic is still unsatisfactory.

Discovery of anti-cancer agents from dietary natural products provides a useful application and chemotherapeutic effectiveness on cancer cells (5), and many studies have focused on killing cancer cells through the promotion of cell cycle arrest and induction of apoptosis (6,7). The protein levels of CDK1 with cyclin B play vital roles in the regulation of G2/M phase (8,9). Otherwise, apoptotic cell death is important after tumor cell exposure to bioactive dietary natural compound, phytochemicals or anti-cancer drugs (10-12).

Apoptotic cell death is divided into two major signaling pathways. The death receptor pathway (extrinsic) begins outside the cell through the activation of the cell surface (Fas/FasL or TRAIL) and then promotes caspase-8 activation (13,14). In the mitochondrial pathway (intrinsic), death signals to mitochondria result in the release of mitochondrial intermembrane proteins such as cytochrome *c*, which associate with apoptotic protease-activating factor 1 (Apaf-1) and pro-caspase-9 to form the apoptosome and then active caspase-3 leading to cell apoptosis (14,15). In addition, the caspase-independent pathway is involved in the mitochondria which leads to release of AIF or Endo G from mitochondria causing cell death (16,17).

Isothiocyanates (ITCs) are present as glucosinolates in various cruciferous vegetables. Allyl isothiocyanate (AITC) is one the common naturally occurring ITCs (18). Many studies have found that AITC can inhibit cancer cell proliferation by causing cell cycle arrest and/or induction of apoptosis, including human leukemia, bladder cancer, cervical cancer and colorectal cancer cell lines (19-22).

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Key words: allyl isothiocyanate, human brain malignant glioma GBM 8401 cells, apoptosis, mitochondria, G2/M arrest

However, no previous study exists addressing whether the AITC inhibits cell proliferation, promotes cell cycle arrest and induces apoptosis in human brain malignant glioma cells. The aim of the present study was to determine and explore AITC-triggered G2/M phase arrest and cell death in GBM 8401 cells via a mitochondria-dependent apoptotic signaling.

Materials and methods

Chemicals and reagents. Allyl isothiocyanate (AITC), propidium iodide (PI), Triton X-100, RNase A, Tris-HCl and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL/Invitrogen Corp. (Grand Island, NY, USA). Caspase-9 inhibitor (Z-LEHD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) (R&D Systems, Minneapolis, MN, USA) were dissolved in DMSO and diluted in cell culture medium before use.

Cell culture. Human brain malignant glioma cell line (GBM 8401) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm² tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and grown at 37°C under a humidified 5% CO₂ and 95% air at one atmosphere. Subconfluent cells were passaged with a solution containing 0.25% trypsin and 0.02% EDTA (23).

Cell morphological examination. About 2x10⁵ cells/well of GBM 8401 cells in a 12-well plate were treated with 0, 10, and 20 µM of AITC and then were incubated for 24 and 48 h. Cells were directly examined and were photographed under contrast-phase microscope (24).

Determination of cell viability by MTT assay. About 1x10⁴ cells/well of GBM 8401 cells in 96-well plates were exposed to 0.5, 1, 5, 10, and 20 µM of AITC, and DMSO, 0.1% in media served as a vehicle control. After a 24 h-incubation, 100 µl of MTT (0.5 mg/ml) solution was added to each well, and the plate was incubated at 37°C for 3 h. An aliquot (100 µl) of 0.04 N HCl in isopropanol was added and the absorbance at 570 nm was measured for each well. The cell survival ratio was expressed as % of control (25).

Cell cycle distribution analysis. The GBM 8401 cells were seeded onto 12-well culture plates at 2x10⁵ cells/well then incubated with 10 µM of AITC for 24 h. The cells were harvested and washed by centrifugation. For cell cycle and apoptosis determination, cells were fixed by 70% ethanol in -20°C overnight and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase A and 0.1% Triton X-100 in dark room for 30 min. The cell cycle distribution and apoptotic nuclei were determined by flow cytometry (FACSCalibur™, Becton Dickinson, Franklin Lakes, NJ, USA) (26).

Assessment of apoptosis morphology by DAPI staining. Approximately 2x10⁵ cells/well of GBM 8401 cells in a 12-well plate were treated with 0, and 10 µM of AITC and 0.1% DMSO as the control. Cells were incubated and grown for 24 h. Cells were stained by 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes/Invitrogen Corp., Eugene, OR, USA) and were examined and photographed under fluorescence microscopy (24).

CDK1 kinase assay. According to the protocol of Medical and Biological Laboratories CDK1 kinase assay kit (MBL, Nagoya, Japan). About 1x10⁷ cells were suspended in a buffer containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin, 10 mM 2-mercaptoethanol, 1 mM MgCl₂, 2 mM EGTA, 0.5 mM dithiothreitol, 0.01% Brij35, 25 mM *b*-glycerophosphate, and 0.5 M NaCl. Cell suspensions were sonicated and centrifuged at 10,000 x g for 30 min. To determine the CDK1 kinase assay condition using MV Peptide and determined by measuring OD 492 as described previously (27,28).

Western blot analysis. About 1x10⁷ cells of GBM 8401 in a 75-T flask were treated with 10 µM of AITC for 0, 6, 12, 24 and 48 h. The examined cells were harvested and washed with cold PBS for detecting the changes of protein levels correlated with G2/M phase-modulated and apoptotic signaling. The total proteins were collected from AITC-treated GBM 8401 cells before the CDK1, cyclin B and cyclin A were detected and measured as previously described (28). The level of cytosolic fraction proteins such as cytochrome *c*, Apaf-1, pro-caspase9, AIF, and Endo G were determined and isolated according to the manufacturer's protocol (Mitochondria/Cytosol fractionation kit, BioVision, Inc. Mountain View, CA, USA). GBM 8401 cells after exposure to AITC were harvested, disrupted and then centrifuged to isolate cytosolic fractions as described elsewhere (24,29). The total or cytosolic protein (40 µg) from each sample was resolved on 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The blot was soaked in blocking buffer (5% non-fat dry milk/0.05% Tween-20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then incubated with anti-CDK1, anti-cyclin B, anti-cyclin A, anti-cytochrome *c*, anti-Apaf-1, anti-pro-caspase-9, anti-AIF, and anti-Endo G antibodies (Santa Cruz Biotechnology, Inc.) in blocking buffer at 4°C overnight. A secondary antibody horseradish peroxidase conjugate was added and detected by chemiluminescence and autoradiography using X-ray film. To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

Assays for caspase-3, -8 and -9 activities. Approximately 1x10⁷ cells of GBM 8401 in a 75-T flask were pretreated with or without the selective inhibitors (Z-DEVE-FMK for caspase-3, Z-IETD-FMK for caspase-8 and Z-LEHD-FMK for caspase-8) and then treated with 10 µM AITC were incubated for 24 h to detect caspase-3, -8 and -9 activities. Caspase-3, -8 and -9 activities were assessed according to the manufacturer's instructions (Caspase colorimetric kit, R&D

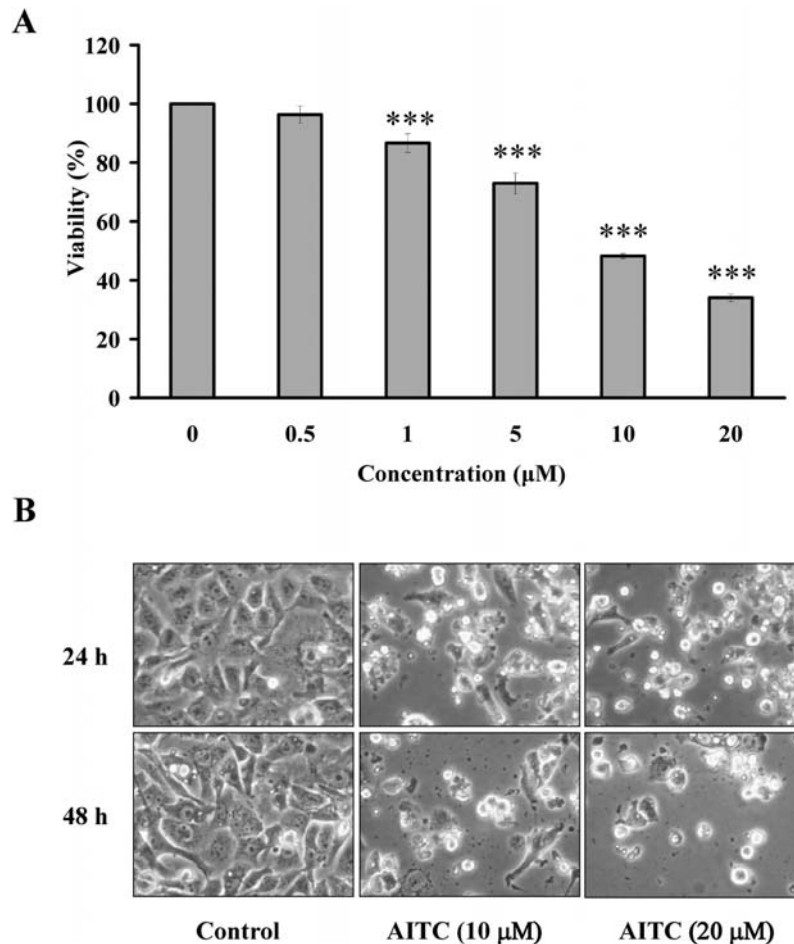


Figure 1. Effects of AITC on cell viability (A) and morphology (B) of GBM 8401 cells. Cells were cultured with 0, 0.5, 1, 5, 10 and 20 μM of AITC for 24 h. Viable cells were determined by MTT assay. The cell morphological changes were examined and photographed under phase-contrast microscopy (x200) as described in Materials and methods. There was a significant difference (***) $P < 0.001$) when compared with the control group by Student's t-test analysis.

System Inc.). Briefly, cells were harvested and lysed in 50 μl lysis buffer containing 2 mM DTT for 10 min. After centrifugation, the supernatants containing 100 μg proteins were incubated with caspase-3, -8 and -9 substrate in reaction buffer. Samples were incubated in a 96-well flat bottom microplate at 37°C for 1 h. Levels of released pNA (Z-DEVD-pNA, Z-IETD-pNA and Z-LEHD-pNA for caspase-3, -8 and -9, respectively) were measured with an ELISA reader (Anthos Labtec Instruments) at OD 405 nm wavelength (27,30).

Statistical analysis. Student's t-test was used to analyze the differences between the AITC-treated and control groups. All data were expressed as mean \pm SD from at least three independent experiments. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were indicative of significant difference.

Results

Cytotoxic effects and morphological changes of AITC on human brain malignant glioma GBM 8401 cells. The potential cytotoxicity of AITC on a human brain malignant glioma cell line GBM 8401 was investigated by the MTT assay. The results are presented in Fig. 1A showing that

AITC significantly reduced the percentage of viable cells as compared to control cells and these effects were dose-dependent. The IC_{50} of AITC was $9.25 \pm 0.69 \mu\text{M}$ after 24 h-treatment. Also, the examined cells exhibited morphological changes, including rounding and shrinkage after 24 and 48 h-incubation with 10 and 20 μM of AITC as shown in Fig. 1B.

Effects of AITC on cell cycle progression and apoptotic cell death. AITC induced reduction of cell viability could be due to apoptosis (sub-G1 population) mediated by cell cycle arrest which were determined. Representative profiles of cell cycle progression and percentage of each phase are presented in Fig. 2A. Significant arrest was seen at G2/M phase at 10 μM of AITC and it significantly increased apoptotic cells (sub-G1 groups) (Fig. 2A). It can be seen in Fig. 2B that apoptotic chromatinistic (nuclear and chromatin condensation) in GBM 8401 cells occurred with or without AITC (10 μM) treatment for 24 h determined by DAPI staining.

AITC modulated relative regulated protein levels of G2/M phase. In order to confirm AITC-induced an accumulation of G2/M population in GBM-8401 cells and it was regulated associated proteins. GBM 8401 cells after exposure to AITC

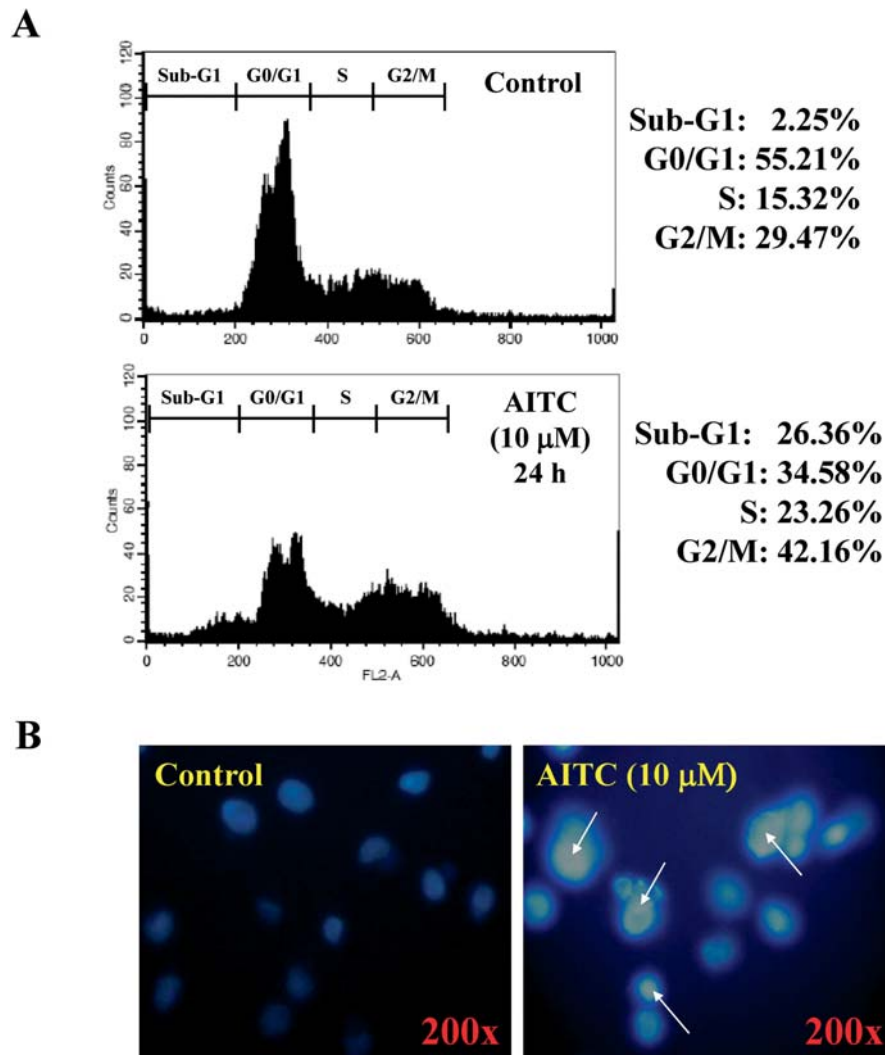


Figure 2. Effects of AITC on cell cycle distribution (A) and chromatin condensation (B) in GBM 8401 cells. Cells were treated with 10 μ M of AITC for 24 h and then were analyzed and determined for cell cycle distribution by PI staining and flow cytometric analysis. AITC induced chromatin condensation (an apoptotic characteristic) in GBM 8401 cells. Cells were incubated with 10 μ M of AITC for 24 h and apoptotic cells were determined by DAPI staining and were photographed by fluorescence microscopy (x200) as described in Materials and methods.

showed that a significant decrease of CDK1 activity (Fig. 3A) and down-regulated the protein levels, including CDK1, cyclin B and cyclin A as shown in Fig. 3B.

AITC enhances caspase-3 and -9 activities and stimulates the associated mitochondrial apoptotic signaling proteins. To further confirm that AITC-induced apoptotic cell death, the results of Western blot analysis from 10 μ M of AITC co-incubation with GBM 8401 cells for 0, 6, 12, 24 and 48 h are shown in Fig. 4A. The increasing protein levels of cytochrome *c*, Apaf-1, pro-caspase-9, AIF, and Endo G in cytosolic fractions may contribute to the occurrence of AITC-triggered mitochondrial apoptosis and this signaling was time-dependent. The results are shown in Fig. 4B, indicating that AITC induced a significant rise in caspase-3 and -9 activities rather than influence of caspase-8 response. This stimulation of activity of the caspase cascade in GBM 8401 cells was reduced after pre-incubation with specific inhibitors of caspase-3 and -9, respectively. Based on these results, AITC-induced cell death was correlated with caspase-dependent and -independent mitochondrial apoptotic pathways.

Discussion

Isothiocyanates (ITCs) have been reported to have anti-cancer effects in both *in vitro* and *in vivo* experimental models (31-33). AITC, also known as mustard oil and existing in dietary cruciferous vegetables, is one of the common naturally occurring isothiocyanates (ITCs) (34,35). It is demonstrated that AITC promoted cell cycle arrest (36,37) and induced apoptosis (34,38) in many types of cancer cells. Therefore, AITC may have potential as dietary chemoprevention/treatment agent (34). However, there is no information on AITC in having anti-cancer activity of human brain malignant glioma cell line and the *in vitro* study of brain cancer GBM 8401 cells is lacking. In this study, we investigated AITC triggered G2/M phase arrest and apoptosis in GBM 8401 cells. We showed that AITC: 1) induced morphological changes; 2) decreased the percentage of viable cells; 3) increased G2/M phase arrest; 4) stimulated the levels of caspase-9, -3, AIF and Endo G through a mitochondria-dependent apoptotic pathway. We examined the effects of AITC on the survival of GBM 8401 cells and our results

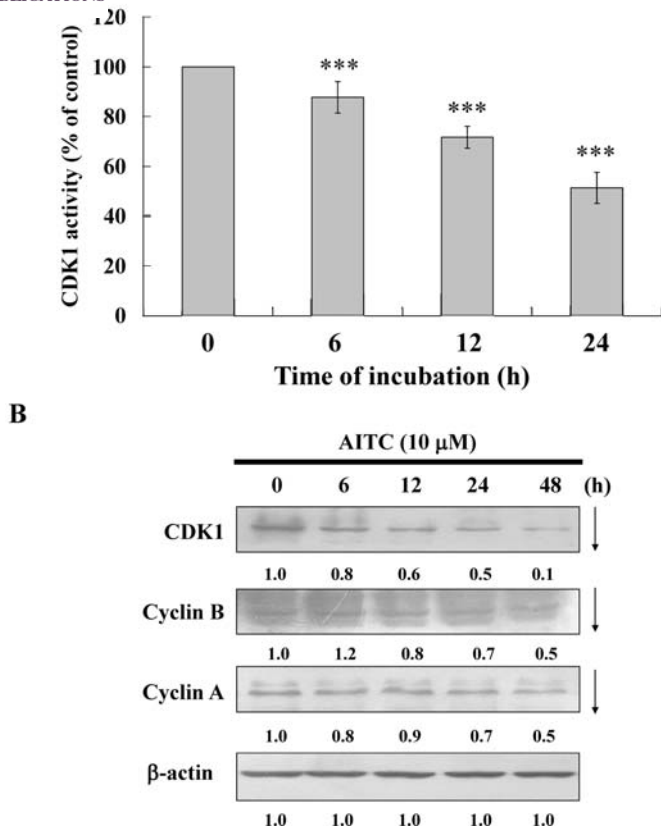


Figure 3. AITC alters the levels of CDK1 activity (A), and the CDK1, cyclin A and cyclin B proteins of G2/M phase arrest in GBM 8401 cells. Cells were incubated with 10 μ M AITC for 0, 6, 12, and 24 h for CDK1 activity. GBM 8401 cells were incubated with 10 μ M AITC for 0, 6, 12, 24 and 48 h, harvested from each sample and then relative protein levels were determined by Western blot analysis. The levels of CDK1, cyclin B and cyclin A were examined, and β -actin as an internal control using SDS-PAGE and Western blotting as described in Materials and methods. ***P<0.001, significantly different compared to the control group.

showed that AITC induced cytotoxic responses in a dose-dependent manner. Also, AITC stimulated morphological changes such as rounding and shrinkage in GBM 8401 cells after 24 and 48 h-treatment.

Cell growth and proliferation of mammalian and tumor cells occur through cell cycle progression. The inhibition of cell cycle distribution has been recognized as a target for anti-cancer agents (39,40). The results in the present study show that GBM 8401 cells were treated with 10 μ M of AITC for up to 24 h predominantly accumulated in the G2/M phase by flow cytometric analysis, suggesting the sequential events of cell cycle arrest followed by apoptosis. This finding agrees with another study on causing G2/M phase arrest in human prostate cancer cells (36). Furthermore, it is reported that the CDK1/Cyclin B complex is one of the major regulators leading the G2 to M progression or apoptosis (24). We investigated the GBM 8401 cells after AITC treatment determining the G2/M phase regulated protein levels and the data showed that a decrease in the protein levels of CDK1, cyclin B and cyclin A by Western blot analysis, and CDK1 kinase assays revealed reduction of CDK1 activity at 6, 12 and 24 h after 10 μ M of AITC treatment.

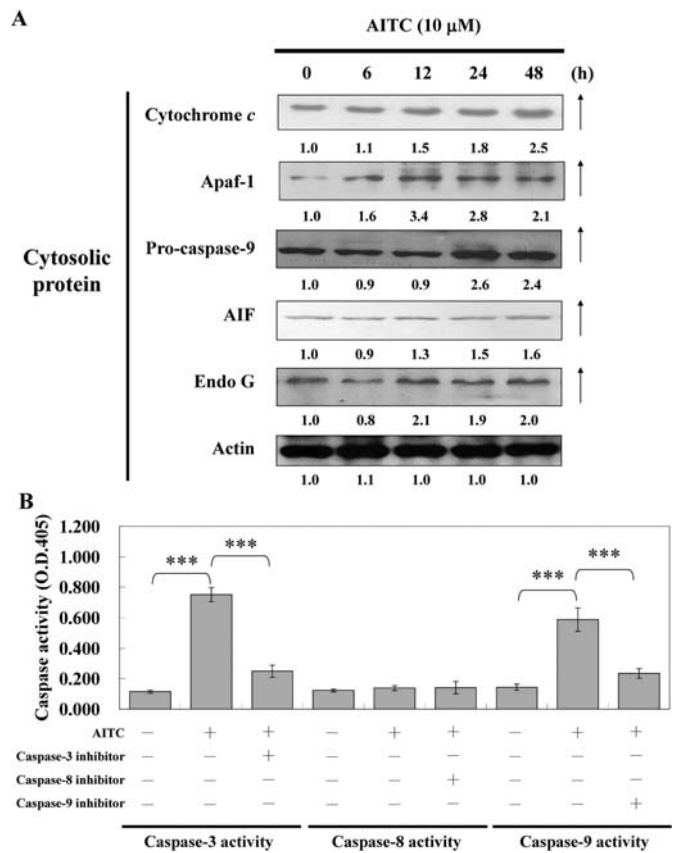


Figure 4. AITC affects the relative protein levels of apoptosis (A) and caspase-3, -8 and -9 activities (B) in GBM 8401 cells. Cells were incubated with 10 μ M AITC for 0, 6, 12, and 24 h then harvested from each sample, and the cytosolic proteins were prepared then detected by Western blotting. The level expressions of cytochrome c, Apaf-1, pro-caspase-9, AIF and Endo G were examined using SDS-PAGE and Western blotting. Cells were preincubated with or without inhibitors of caspase-3 (Z-DEVE-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK), respectively for 3 h and then treated with 10 μ M AITC for 24 h. AITC stimulated the activities of caspase-3 and -9 of GBM 8401 cells and it was determined as described in Materials and methods. ***P<0.001, significant difference.

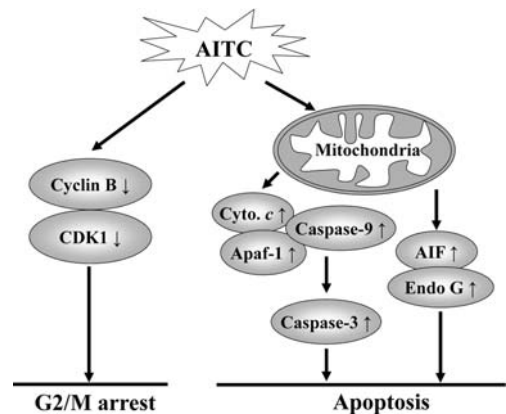


Figure 5. The proposed model of AITC-mediated G2/M phase arrest and apoptosis in human brain malignant glioma GBM 8401 cells.

Induction of apoptotic cell death is one of the best strategies for cancer treatment (10,41). Our results indicated that AITC induced apoptosis in GBM 8401 cells as demonstrated by

flow cytometric analysis and DAPI staining was applied to confirm cell apoptosis in examined GBM 8401 cells. These results corroborate findings of other studies on several different cancer cell lines (34,36-38,42). Furthermore, apoptotic cell death is a genetically regulated biological process and it can be divided into extrinsic and intrinsic signaling major pathways (43,44). The members of Bcl-2 family act as repressors of apoptosis and mitochondrial dysfunction by releasing of apoptotic factors such as cytochrome *c*, apoptosis-inducing factor (AIF), and endonuclease G (Endo G), cytochrome *c*, once released into the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 pro-enzymes. Active caspase-9 activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to cell apoptosis through mitochondria-dependent pathway (45). The AIF and Endo G also triggered apoptosis through a caspase-independent pathway (46). The results from the Western blot analysis showed that AITC increased protein levels of cytochrome *c*, Apaf-1, pro-caspase-9, AIF, and Endo G, suggesting that analyses of protein abundance indicated that AITC induced apoptosis through a mitochondria-dependent pathway. Also, AITC stimulates the loss of mitochondrial membrane potential ($\Delta\Psi_m$) resulting from the mitochondria dysfunction (data not shown). The activation of the caspase cascade is the major mechanism that promotes apoptosis in response to death-inducing signals from mitochondria stress (43,47). In this study, we observed the AITC induced caspase-9 and -3 activities in GBM 8401 and the specific inhibitors (Z-DEVE-FMK for caspase-3 and Z-LEHD-FMK for caspase-9) individually prevented AITC-induced caspase-3 and -9 activities, respectively in GBM 8401 cells.

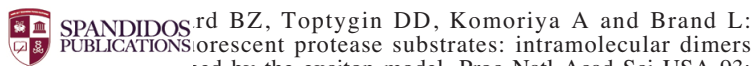
The schematic proposed model of AITC-mediated G2/M phase arrest and apoptosis in human brain malignant glioma GBM 8401 cells is shown in Fig. 5. In conclusion, AITC mediated G2/M phase arrest and induced apoptosis in human brain malignant glioma GBM 8401 cells. AITC decreased the regulated protein levels of cyclin B and CDK1 and then led to G2/M phase arrest. AITC-stimulated apoptotic cell death resulted in the alteration of a series of mitochondrial dysfunction and apoptotic signaling. AITC increased the activity of caspase-9, and -3, and the release of mitochondrial cytochrome *c*, AIF and Endo G leading to cell apoptosis.

Acknowledgements

The investigation was supported by a research grant from the National Science Council of the Republic of China (NSC 96-2321-B-039-007) and grants from China Medical University, Taichung, Taiwan (CMU96-114).

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